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*THE EFFECTS OF STREPTONIGRIN ON CULTURED HUMAN LEUKOCYTES**

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Streptonigrin (SN) is a derivative of *Streptomyces flocculus* which has been isolated and screened for its possible antitumor and antibiotic activity. It has not been released for clinical use execpt under experimental conditions. Studies with rodents indicated that it is an extremely cytotoxic compound.[1]

Recent work in this laboratory has shown that SN induces the production of bacteriophage in lysogenic bacteria while inhibiting the net synthesis of bacterial deoxyribonucleic acid (DNA).[2] Furthermore, it causes a marked increase in genetic recombination in mixed bacteriophage infections,[3] and initiates rapid *in vivo* degradation of the DNA of *Escherichia coli*.[4]

Because of these striking effects on macromolecular synthesis, tests of the

activity of SN on human chromosomes seemed warranted. The results to be presented in this communication indicate that, even in extreme dilution, SN induces chromosomal breakage in cultured human leukocytes.

Materials and Methods.—Streptonigrin ($C_{24}H_{20}O_8N_4$), in dry powder form, was obtained from C. Pfizer and Co., Brooklyn, New York. The powder was dissolved in acetone (1 mg/ml) and dilutions were made with sterile distilled water. Various concentrations were added to human leukocyte cultures which had been prepared according to a method similar to that of Moorhead *et al.*[5]

Preliminary experiments, using blood from ten normal individuals, indicated that SN had profound effects on cell growth and chromosome structure. To test the effects of various concentrations for different periods of exposure, the following experiment was performed. Twenty-four treated cultures and two control cultures were established with plasma (30 ml) obtained by a single venipuncture from a 28-year-old normal male. The effects of three concentrations and seven exposure periods were studied according to the regimen outlined in Table 1. Since cells incubated with 0.1 μg/ml for more than 12 hr were destroyed, longer exposure times at this concentration were not tested. Furthermore, since the preliminary work had shown total cell destruction when cultures were treated for the entire 72 hr incubation period with concentrations of SN as low as 0.0001 μg/ml, the maximum period of exposure was limited to 36 hr.

TABLE 1

THREE CONCENTRATIONS OF STREPTONIGRIN INCUBATED WITH LEUKOCYTE CULTURES FOR VARIOUS LENGTHS OF EXPOSURE PRIOR TO HARVEST OF THE CELLS

Concentration of SN	Hours exposed before harvest of culture						
(μg/ml)	2	6	12	18	24	30	36
0.001	−	++	−	++	++	++	++
0.01	++	++	+	++	++	++	−
0.1	+	+	+	−	−	−	−

++ Replicate cultures.
+ Single culture.
− No culture.

Three slides per culture were prepared, coded, and examined by observers who had no knowledge of the treatment. For each slide, the mitotic rate was computed on the basis of the number of division figures observed per 1,000 cells. Ten metaphase cells per slide, selected for adequate chromosome spreading under low power (250×), were analyzed in detail for chromosome number and morphology using oil immersion, phase contrast microscopy (2,500×). Due to the low mitotic rate of the 12 hr exposure to 0.1 μg/ml SN, only 11 analyzable metaphase cells could be found. Each cell thus chosen was scored independently by at least two observers for the presence of chromosomal abnormalities. Only those breaks scored by two observers were counted. Following this initial scoring, 15 additional cells per control slide were examined, bringing the total to 150 control cells analyzed. Chromosomal abnormalities were assigned to seven groups according to the Denver Classification,[6] and where possible, to individual chromosomes. Also, attempts were made to score breaks according to specific regions of the chromosome. A few breaks, rearrangements, and fragments could not be classified.

Results.—Mitotic rate: Statistical analysis of results from replicate cultures exposed to the same treatment showed no significant differences in mitotic rates;

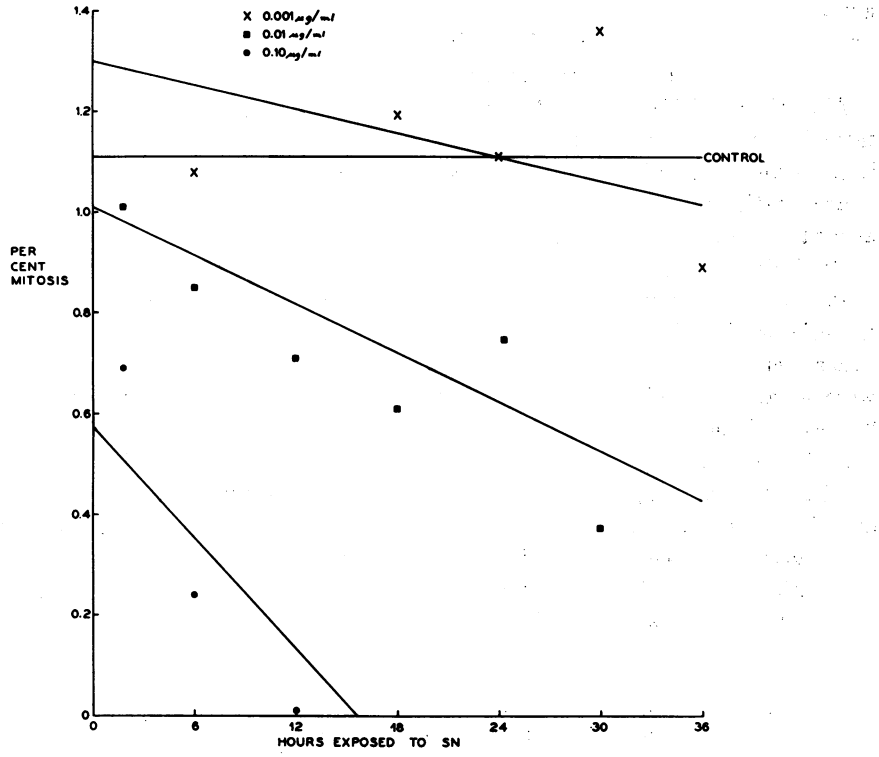

FIG. 1.—The effect on mitotic rate with three concentrations of SN for varying lengths of exposure. The curves drawn are least square lines calculated by regression analysis; each point indicates the average mitotic rate for approximately 6,000 cells.

therefore, these data were pooled. The effect of SN as an inhibitor of mitosis appears related to both concentration and length of exposure (Fig. 1). The control cultures had a mitotic rate of 1.12 per cent. Regression analysis indicated that the mitotic rate of cultures exposed to SN at a concentration of 0.001 μg/ml for 36 hr or less did not differ significantly from that of the controls. However, the mitotic rates of cells exposed to 0.01 and 0.1 μg/ml SN were significantly below the control rates.

Chromosome damage: SN caused much chromosomal breakage and rearrangement. The distribution of chromosome counts in those cells examined in detail appears in Table 2. A modal number of 46 chromosomes was observed in 95.3 per cent of the treated cells. The fact that some cells (3.3 per cent) contained less than 46 chromosomes may be due to random loss of chromosomes caused by rupture of the cell membrane in the preparation of the slide. Counts of greater than 46 chromosomes (1.4 per cent) may be partially attributable to random gain by the

TABLE 2

CHROMOSOME COUNTS IN CELLS EXAMINED FOR CHROMOSOMAL DAMAGE AFTER TREATMENT WITH STREPTONIGRIN

Chromosome number / Number of cells	41	43	44	45	46	47	48	49	Acentric fragments	Total cells
Treated	1	1	4	17	668	6	2	2	46	701
Control	1		2	7	138	2			1	150

breakage of chromosomes with the emergence of fragments or the inclusion of chromosomes from broken cells. Acentric fragments were observed in 46 of the 701 treated cells. The following types of chromosomal abnormalities were produced by SN:

1. Chromatid break—a visual discontinuity of the chromatin material in one chromatid with no apparent strand connecting the broken ends, regardless of the alignment of the two segments (Fig. 2*a*).
2. Isochromatid break—breaks at the same position in both sister chromatids (Fig. 2*b*). Throughout this study isochromatid breaks were scored as a single break.
3. Acentric fragment (Fig. 2*a*).
4. Dicentric chromosome.
5. "Translocation" cross configuration (Fig. 2*c*).
6. Subtelomeric (end-to-end) association of chromosomes (Fig. 2*c*).
7. Cleavage or severe attenuation of the centromere region (Fig. 2*d*).
8. Fusion of the ends of the chromatids of a single chromosome.
9. Increased "stickiness" as evidenced by chromatin strands between chromosomes (Fig. 2*c*).
10. Uncoiling of the chromosomes with long attenuated segments (Fig. 2*d*).
11. Severe fragmentation or degeneration of the entire chromatin material (Figs. 2*e*, 2*f*).

The detailed distribution of breaks by chromosome groups is given in Table 3. A total of 395 breaks and rearrangements of various types was seen in 701 treated cells (0.563 breaks/cell). Four chromatid breaks were observed in the 150 control

TABLE 3

DISTRIBUTION OF CHROMATID AND ISOCHROMATID BREAKS ACCORDING TO THE DENVER CLASSIFICATION OF CHROMOSOME GROUPS

Treatment	Number of breaks by group*								No. of cells examined	Mean number of breaks per cell
	1–3	4–5	6–12, X	13–15	16–18	19–20	21–22, Y	Total		
Control	3				1			4	150	0.027
0.001 μg/ml SN										
6 hr	8	4	6					18	60	0.300
18 hr	5	2	7	1	1			16	60	0.267
24 hr	5	4	9	3	3			24	60	0.400
30 hr	4	4	7	1	2			18	60	0.300
36 hr	4	1	3		1			9	60	0.150
Total:	*26*	*15*	*32*	*5*	*7*			*85*	*300*	*0.283*
0.01 μg/ml SN										
2 hr	4		3					7	60	0.117
6 hr	5	4	5	2	2		1	19	60	0.317
12 hr	7		6	2	1			16	30	0.533
18 hr	10	9	14		4	1	2	40	60	0.667
24 hr	23	13	35	11	2		1	85	60	1.417
30 hr	12	9	29	7	6	1	1	65	60	1.083
Total:	*61*	*35*	*92*	*22*	*15*	*2*	*5*	*232*	*330*	*0.703*
0.1 μg/ml SN										
2 hr	4	2	9		1			16	30	0.533
6 hr	8	3	9		4			24	30	0.800
12 hr	12	4	10	2	3			31	11†	2.818
Total:	*24*	*9*	*28*	*2*	*8*			*71*	*71*	*1.000*
Total	111	59	152	29	30	2	5	388		

* Does not include 7 unclassified breaks.
† Due to low mitotic rate only 11 analyzable cells found.

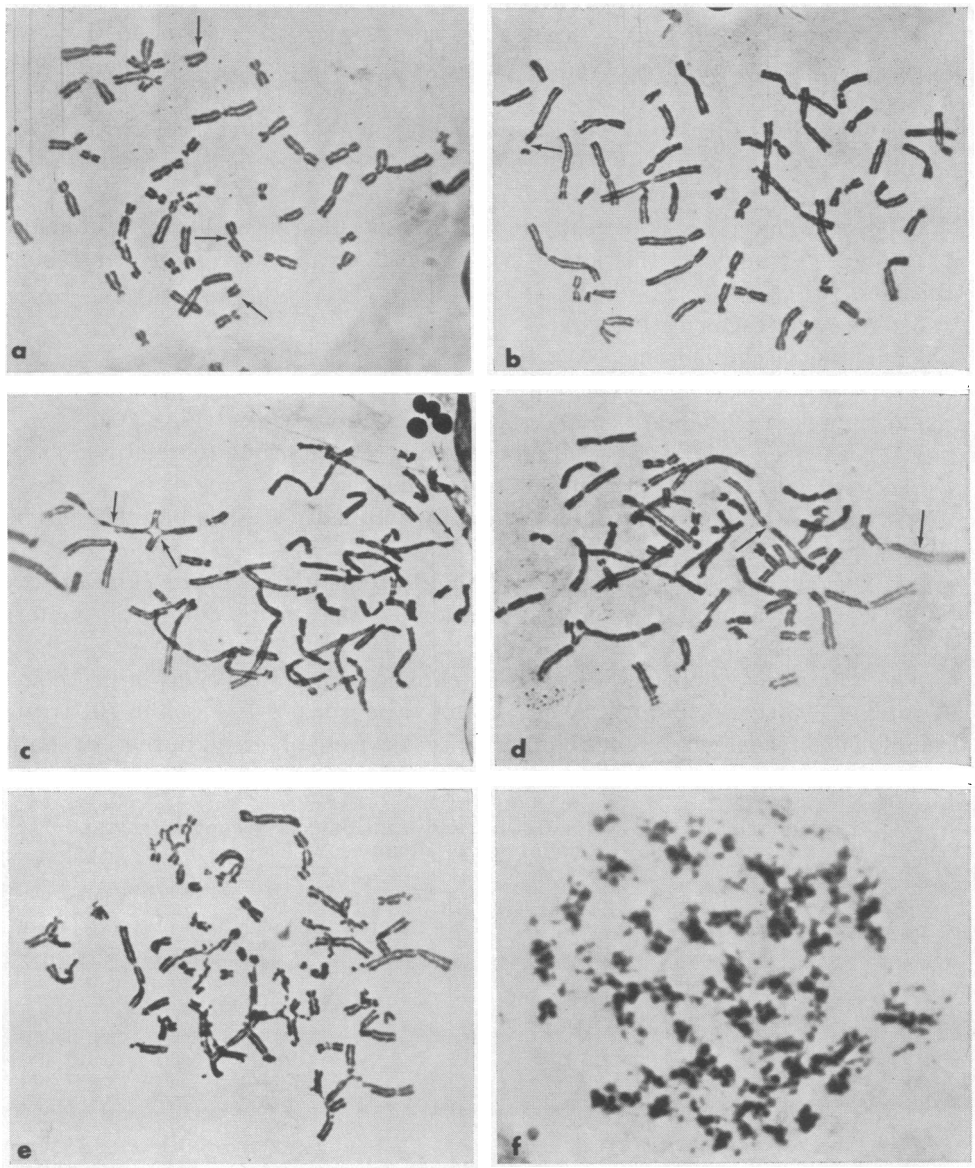

FIG. 2.—Metaphase cells illustrating types of chromosomal damage due to SN. Cells *a* through *e* were treated with 0.01 μg/ml of SN for 24 hr, while cell *f* was treated with 0.1 μg/ml of SN for 6 hr. Arrows indicate type of chromosome damage: (*a*) chromatid break and acentric fragments; (*b*) isochromatid break; (*c*) "translocation" cross, "end-to-end" fusion, and chromatin strand; (*d*) centromere attenuation and uncoiled chromosome segment; (*e*) and (*f*) fragmentation and degeneration of chromosomes.

cells (0.027 breaks/cell). This latter figure compares with frequencies of 0.016 and 0.024 spontaneous breaks per cell in leukocyte cultures reported by Bender and Gooch.[7, 8]

Effects of concentration and length of exposure: Both the mean number of breaks per cell and the percentage of cells with breaks appear to increase with increasing concentrations of SN (Fig. 3). The mean number of breaks per cell was 0.28,

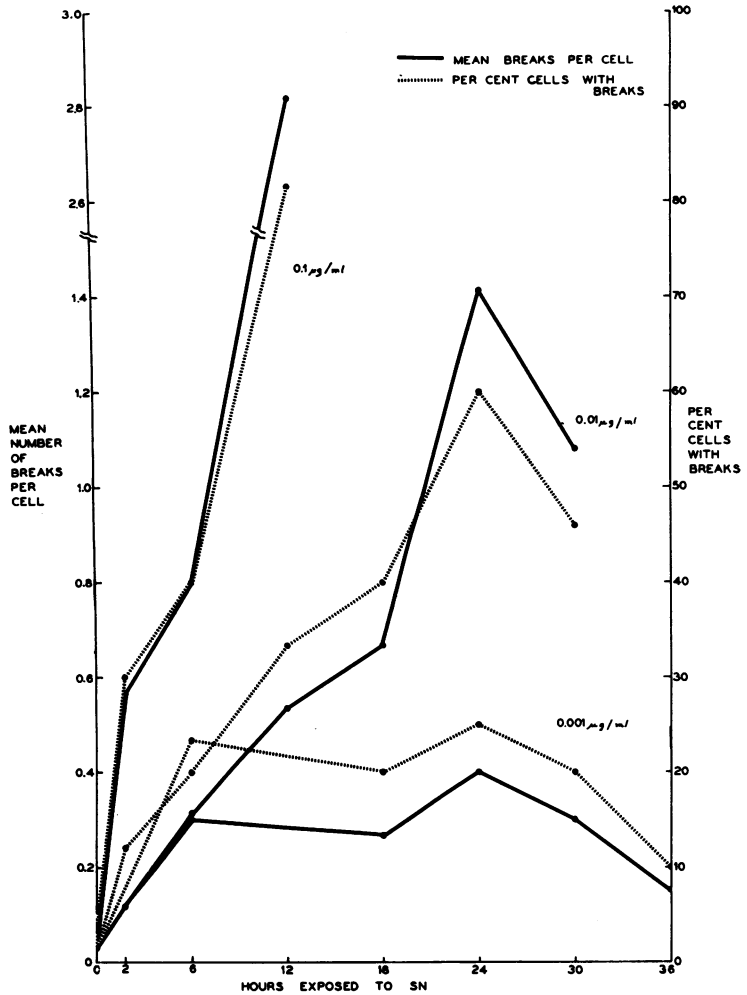

FIG. 3.—The distribution of mean breaks per cell and per cent cells with breaks due to three concentrations of SN for various exposure times.

0.70, and 1.00 for concentrations of 0.001, 0.01, and 0.1 μg/ml of SN, respectively, while 19.7, 35.1, and 42.2 per cent of the cells showed breaks at these same concentrations. An increased number of abnormalities was also observed upon lengthened exposure times. The decrease in breaks following exposures longer than 24 hr is not statistically significant.

Specificity of action: From the distribution in Table 3, SN does not appear to break the chromosomes randomly according to the relative amounts of chromatin material in each of the groups (Table 4: $\chi^2 = 32.19$, $P < 0.005$, df = 6). The expected number of breaks in each group was derived from the mean length of each chromosome expressed in units according to the measurements given in the Denver report.[6] The mean lengths of the X and Y chromosomes were added to the doubled mean lengths of the 22 autosomes to obtain the total chromatin material in the normal male somatic chromosome complement. If chromosomal breaks were ran-

TABLE 4

"GOODNESS OF FIT" TEST FOR RANDOM DISTRIBUTION OF CHROMOSOME BREAKS, BASED ON UNIT CHROMOSOME LENGTHS GIVEN IN THE DENVER REPORT[6]

Chromosome group	1–3	4–5	6–12, X	13–15	16–18	19–20	21–22, Y	Total
Observed	111	59	152	29	30	2	5	388
Expected	92.68	47.63	140.54	39.46	33.69	17.79	16.21	388.00
χ^2	3.62	2.71	0.93	2.77	0.40	14.01	7.75	32.19

$P < 0.005$; df = 6.

domly distributed according to length, the expected number of breaks for each group would be obtained by the product of the total number of breaks observed and the relative per cent chromatin in each chromosome group. A deficiency of breaks was noted in the chromosomes of groups 19–20 and 21–22, Y. Only seven breaks were found in these chromosomes, whereas a total of 34 would be expected. No abnormalities were seen in any of the Y chromosomes. Investigation of the randomness of breaks among chromosomes other than the 19–20 and 21–22, Y groups, by assignment of breaks to specific regions of chromosomes which can be individually recognized, is in progress. Additional cells must be scored and precise measurements made before the results are interpreted.

Discussion.—Most of the antibiotics now in widespread clinical use are derived from various species of Streptomyces (e.g., the tetracyclines, erythromycin, mycostatin, and chloramphenicol). It has been shown that certain Streptomyces-derived antibiotics have profound inhibitory influences on the synthesis of nucleic acids and protein. Mitomycin C inhibits bacterial DNA synthesis[9, 10] and its presence in the bacterial cultures leads to a rapid breakdown of DNA to acid-soluble fragments.[11, 12] Actinomycin D depresses mitosis and the metabolism of DNA, RNA, and protein.[13–15] These data, in conjunction with the results presented above, indicate the need for careful studies of the effects of this class of compounds on chromosomal integrity.

Streptonigrin is a recent and exciting addition to the class of antibiotic antimetabolites. The high frequency of chromosomal abnormalities obtained with the minute concentrations used in this study indicates the extreme potency of this agent. Dosages of SN as low as 2.0×10^{-7} *M* caused complete death of the culture in 72 hr, while 2.0×10^{-9} *M* caused 2.82 breaks/cell during a 12 hr exposure. This is in contrast to the effects of 5-bromodeoxyuridine on strain L-M cells in long-term tissue culture, where treatment for 28 days with concentrations of 8.1×10^{-5} *M* produced 1.98 breaks/cell.[16] Changes in chromosomal architecture, like those observed in this study, would most likely lead to reduced viability, if not death of the cell. Inhibition of mitosis and lethal chromosome effects may well be manifestations of the antitumor activity ascribed to SN.

Recently, mammalian chromosome damage of a specific nature has been induced by 5-bromodeoxyuridine[16] and hydroxylamine.[17] The action of SN in causing chromosomal breaks is also nonrandom in the sense that it is not related to unit chromosomal lengths. A relative stability of the chromosomes in groups 19–20 and 21–22, Y, is apparent from the significant deficiency of breaks in these chromosomes. This finding may be partially explicable by increased difficulty in detecting breaks in these smaller chromosomes. Generally, breaks and con-

strictions in chromatids were more frequently visible in those cells with long, uncoiled chromosomes in late prophase or prometaphase (Figs. 2*c*, 2*d*). Upon examining cells with short, compact chromosomes in late metaphase, many breaks seemed either to have "healed" or to have been concealed by the coiling process. This would decrease the probability of identifying breaks in the smaller chromosomes. However, evidence exists which suggests an increased stability of these specific chromosome groups. Karyotypes of aneuploid human cell lines, established from effusions or excised tissues of cancer patients, showed a normal diploid complement for chromosomes of the 21–22, Y group, while the other groups underwent ploidy changes and rearrangements.[18] Karyotypic analyses of a human aneuploid cell line, derived from a biopsy specimen of carcinoma of the cervix, showed disomy of chromosomes 19–20 and 21–22, while all other groups had undergone polysomy and/or rearrangements.[19] This is perhaps surprising in view of the numerous anomalies of chromosome 21 reported in many patients.

The study on genetic recombination in bacteriophage indicates a specific time during the first half of the latent period when SN activity reaches its maximum.[3] Although the present experiment demonstrated an increased effect with increasing exposure times, it would be necessary to design a different experiment to show a specific period of SN activity on leukocytes. Studies utilizing autoradiographic techniques superimposed on SN-treated cultures may be helpful in determining whether the drug acts during the early period of interphase (G_1) or during the period of DNA synthesis (S). Other approaches which may be informative include: (1) "pulsing" of cultures with SN to minimize any possible cumulative effects of long exposures; (2) attempts to synchronize the division of leukocytes. The use of synchronized cultures and/or autoradiographic studies may delineate the specific time of SN activity in the division cycle.

Summary—Streptonigrin is a derivative of *Streptomyces flocculus* with antitumor and antibiotic properties. This drug, when used in extreme dilutions, inhibits mitosis of cultured human leukocytes and causes extensive chromosomal breakage and rearrangements. A variety of chromosomal abnormalities and bizarre metaphase figures were observed. The effect of streptonigrin increased with increasing concentrations as well as lengthening exposure times. Chromosomal damage appears to be nonrandomly distributed with a deficiency of breaks in chromosomes 19, 20, 21, 22, and Y. Approaches to delineate the time of action of streptonigrin in the division cycle are discussed.

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X-CHROMOSOME MOSAICISM IN FEMALES WITH MUSCULAR DYSTROPHY

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Muscular dystrophy of the childhood or classical Duchenne-type is classified by: (1) the onset of muscular weakness in the pelvic girdle and lower limb muscles, usually before the 7th year of life; (2) appearance almost exclusively in male children; (3) presence of enlargement ("pseudo-hypertrophy") in the calves and deltoids; and (4) a rapid progression of weakness to other muscle groups, leading to death by age 15.

The disease is inherited as a sex-linked trait. Males are affected—females rarely so. The carrier state has been detected in the female in some recent studies which utilized appropriate serum enzyme measurements.[1-4] The dystrophic gene is carried on the X-chromosome and usually only manifests itself in the male. Thus, it is interesting that about 40 females with fairly "classical" Duchenne-type dystrophy have been recorded in the literature.[5] These could be explained by: (*a*) the mating of a carrier female with an affected male; (*b*) the mating of a carrier female with a normal male who had undergone mutation on his X-chromosome; and (*c*) a female with an XO-chromosome complement (Turner's syndrome), the mutant gene being on the single X-chromosome and showing full manifestation. Of these suggestions (*a*) is unlikely, since affected males rarely reproduce; (*b*) is very improbable, since Walton[6] has estimated that this may only occur once in 1,000 million population or one case for every 50,000 dystrophic males; and (*c*) remains a possibility and could only be ruled out by chromatin determinations in these patients. One of the female patients described by Walton[6] may have had an XO-chromosome complement.

Forms of juvenile muscular dystrophy which begin in the lower limbs, as proposed by Becker,[7, 8] include (*a*) malignant X-chromosomal type (Duchenne-type),